Acta Biochimica Polonica

Vol. 44 No. 1/1997

89–98

QUARTERLY

The enthalpimetric determination of inhibition constants for the inhibition of urease by acetohydroxamic acid

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Received: 3 May, 1996; revised: 20 August, 1996; accepted: 10 January, 1997

Keywords: urease inhibition, acetohydroxamic acid, enzyme calorimetry

The effect of concentration of acetohydroxamic acid (AHA) on inhibition of jack bean urease in phosphate buffer, pH 7.0, at 25°C, was studied. The measurements were performed at urease concentration of 2.5 mg/100 cm^3 for concentrations of urea and AHA ranging in the range of 2–50 mmol dm^{-3} and 0.25–10 mmol dm^{-3}, respectively. The reactions were monitored by two techniques: analytical and enthalpimetric. For the analytical technique the growth of ammonia concentration in the course of the reaction was determined. From the recorded progress curves the following parameters were calculated for each inhibitor concentration: the initial reaction rate, the steady-state rate and the inversion constant. From these parameters the inhibition constants of the initial and steady-state stages of the reaction, K_i and K_i^*, were calculated. The former constant did not change whereas the latter one proved to decrease quickly with an increase in inhibitor concentration. This behaviour resulted from the fact that the inactive complex EI* was not a product of internal inversion but was formed in the reaction: $\frac{2}{3}I + EI \rightarrow (EI \bullet \frac{2}{3}I)^*$. The dissociation constant of this complex is equal to about 0.3×10^{-3} (mol $dm^{-3})^{2/3}$.

In the 1960s Kobashi *et al.* [1] found that acetohydroxamic acid along with other hydroxamic acids R–CONH–OH ⇔ R-C(OH)=NOH and some of their derivatives are strong and specific inhibitors of plant and microbial ureases [1–3]. They proved that these inhibitors combine with urease stoichiometrically and block the active site with a –CONHOH group. They determined the inhibitory strength of numerous hydroxamates assuming as its measure the concentration of an inhibitor at which the urease activity falls to 50% of its original value.

Subsequent studies on urease inhibition by hydroxamic acids have been reviewed by Mobley & Hausinger [4]. Some of their results indicate that hydroxamic acids could be used in medical therapies.

The enzymatic kinetics of urea hydrolysis in the presence of acetohydroxamic acid (AHA) was a subject of a number of papers [5–9]. Zerner *et al.* [5–8] studied a plant urease and proposed a mechanism which according to Morrison & Walsh [10] was classified as classical fast-binding competitive inhibition. Todd & Hausinger [9] investigated the microbial urease *Klebsiella aerogenes* and found AHA to be a slow-binding competitive inhibitor. This means that urea hydrolysis proceeds along scheme (1).

The aim of this study was to characterize the work of the system in the course of time,

*This work was supported by the State Committe for Scientific Research (KBN, grant No. 3 T09A 072 11.

Abbreviation: AHA, acetylohydroxamic acid.

within the period sufficiently long for the steady-state rate v_s to become visible on the progress curve, and within the inhibitor concentration range sufficiently broad for the relations $1/v_0$, $1/v_s$, k *vs* [I] to become clear. These relations were interpreted in terms of the aforementioned theory of slow-binding competitive inhibition. The progress of urea hydrolysis, i.e. the increase in NH_3 concentration with time, was recorded. Two techniques were applied: enthalpimetric and analytical. The former allowed to measure rates of the reaction at its initial fast stage with satisfactory accuracy. The latter was used for the reactions at high concentrations of the inhibitor; under these conditions a small amount of ammonia was produced and the amount of heat secreted was proportionally low.

Basic equations and symbols

The general model of slow-binding inhibition introduced by Morrison & Walsh [10] covers relations shown in scheme (1):

$$\begin{aligned} &E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_7} E + P \\ &E + I \underset{k_4}{\overset{k_3}{\Leftrightarrow}} EI \underset{k_6}{\overset{k_5}{\Leftrightarrow}} EI^* \end{aligned} \qquad (1)$$

the equation of the progress curve, P *vs* t, has the form (2):

$$P = v_s t + \frac{v_0 - v_s}{\frac{K_i}{[I]}k}\left(1 - e^{-\frac{K_i}{[I]}kt}\right) \qquad (2)$$

The slope of the tangent at $t = 0$ is equal to v_0, whereas the slope of the asymptote is equal to v_s. The half-time, $t_{1/2}$, is the abscissa of the point of intersection of the progress curve with the straight line which divides the intercept of the asymptote into halves and is parallel to the asymptote. The time $t_{1/2}$ is connected with the inversion constant by eqn. (3):

$$k = \frac{\ln 2}{t_{1/2}}\frac{[I]}{K_i} \qquad (3)$$

At high inhibitor concentrations, 4,...,10 mmol dm^{-3} eq. (4) was used:

$$k = \frac{2v_0}{n_{NH_3}}\frac{[I]}{K_i} \qquad (4)$$

where n_{NH_3} denotes final ammonia contents in moles (cf. Table 2.

The following linear relations between variables $1/v_0$, $1/v_s$ and inhibitor concentration appear in the theory of the slow-binding competitive inhibition:

$$\frac{1}{v_0} = \frac{K_m}{K_i v_{max}[S]_0}[I] + \frac{1}{v_{max}}\left(1 + \frac{K_m}{[S]_0}\right) \qquad (5)$$

$$\frac{1}{v_s} = \frac{K_m}{K_i^* v_{max}[S]_0}[I] + \frac{1}{v_{max}}\left(1 + \frac{K_m}{[S]_0}\right) \qquad (6)$$

K_m is the Michaelis constant, K_i and K_i^* are inhibition constants of initial and steady-state stages of the reaction, respectively. The former can also be identified with the dissociation constant of EI complex (eqn. 7), as justified by the fact that the reaction $E + I \Leftrightarrow EI$ reaches equilibrium quickly. On the contrary, the latter one cannot be related to any equilibrium, therefore the term "overall dissociation constant" for K_i^* will not be used in this study. In equations (8) the term K_i^* appears only as a substitute.

$$K_i = [E][I]/[EI] = k_4/k_3 \qquad (7)$$

$$K_i^* = [E][I]/([EI] + [EI^*]) = K_i k_6/(k_5 + k_6) \qquad (8)$$

Symbols:

- k is the inversion constant for the establishment of the equilibrium between EI and EI*,

$$k = k_6\left(1 + \frac{[S]_0}{K_m} + \frac{[I]}{K_i^*}\right) \qquad (9)$$

- k_j is the rate constant related to an elementary reaction j in scheme (1); k_1 and k_3 are in $mol^{-1}\ dm^3\ s^{-1}$, the others in s^{-1},
- v_{max}, v_0 and v_s, in mol s^{-1}, are the maximum reaction rate defined by the Michaelis-Menten equation, initial reaction rate and steady-state reaction rate of

the hydrolysis of urea, respectively, all related to the known constant mass of the enzyme used in the experiment. Other symbols have generally accepted meanings and are expressed in SI units.

Eqn. (5) and (6) allow to verify the hypothesis that acetohydroxamic acid is a competitive slow-binding inhibitor of plant urease.

EXPERIMENTAL

Materials

Jack bean urease was Sigma type III. Its specific activity was 32 units per mg of active protein. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7 and 25°C. Acetohydroxamic acid (Analar grade) was purchased from Sigma. Urea and all other chemicals (Analar grade) were obtained from POCh (Gliwice, Poland).

Enzymatic reaction

The hydrolysis of urea catalyzed by jack bean urease $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$ was studied. All measurements were carried out at enzyme concentration of 2.5 mg/100 cm^3 in phosphate buffer, pH 7.0 (50 mmol dm^{-3}, 1 mmol dm^{-3} EDTA) at 25°C. Jack bean urease is known to reach the highest activity at neutral pH [11].

Reaction without inhibitor. In the first series of buffer solutions, in which the initial urea concentrations were: 2, 3, 5...50 mmol dm^{-3}, the concentrations of ammonia were measured after 2, 3, 4 and 5 minutes of the reaction (analytical method). From these data the initial reaction rates were calculated. The reaction was initiated by addition of 0.25 cm^3 of the urease solution (2.5 mg cm^{-3}) into 25 cm^3 of the assay mixture.

Reaction with inhibitor. In the second series of buffer solutions, urea concentration was kept constant at 50 mmol dm^{-3}, while those of AHA were: 0, 0.5, 1, 1.5 and 3 mmol dm^{-3}. The reactions were carried out in a calorimeter, and temperature increments were recorded as a function of time, ΔT *vs* t.

In the third series of solutions of higher AHA concentration: 4, 5, 6, 8 and 10 mmol dm^{-3}, the reaction was observed in the same conditions by the analytical method. The reactions of both series were initiated by addition of 1 cm^3 of the urease solution (2.5 mg cm^{-3}) into 100 cm^3 of the assay mixture. The obtained two families of progress curves: calorimetric and analytical, were used to calculate the initial reaction rate v_0, steady-state reaction rate v_s and constant k as a function of inhibitor concentration.

Methods

In the calorimetric method the reaction was carried out in the isoperibol set and the rate of temperature change was recorded at 2 s intervals with an accuracy of 0.001°C. The observed differential increments ΔT were corrected for heat exchange into $\Delta T'$. The detailed description of the calorimetric apparatus was presented elsewhere [12].

In the analytical method the concentration of ammonia was determined by the phenol-hypochlorite colorimetric method; the absorbance was measured at 625 nm [13].

RESULTS

Michaelis constant K_m and maximum reaction rate v_{max}

The measured initial rates of urea hydrolysis (in the absence of the inhibitor) as a function of urea concentration form a hyperbola: $v_0 = v_{max}\,[S]_0/(K_m + [S]_0)$ (Fig. 1A). In the Hanes coordinate system [14] this relationship becomes linear (10),

$$\frac{[S]_0}{v_0} = \frac{1}{v_{max}}[S]_0 + \frac{K_m}{v_{max}} \qquad (10)$$

as presented in Fig. 1B. According to the statistical analysis performed by Wilkinson, this linearization procedure is the most reliable of the methods commonly used [15].

From the parameters of the straight line the kinetic constants of the reaction were calculated:

$$K_m = (10.2 \pm 0.5)\ 10^{-3}\ \text{mol dm}^{-3}$$
$$v_{max} = (1.075 \pm 0.02)\ 10^{-6}\ \text{mol s}^{-1}$$

The value of K_m obtained here is higher than that reported previously [12], $6.5 \pm 0.2 \times 10^{-3}$, because it has been determined at

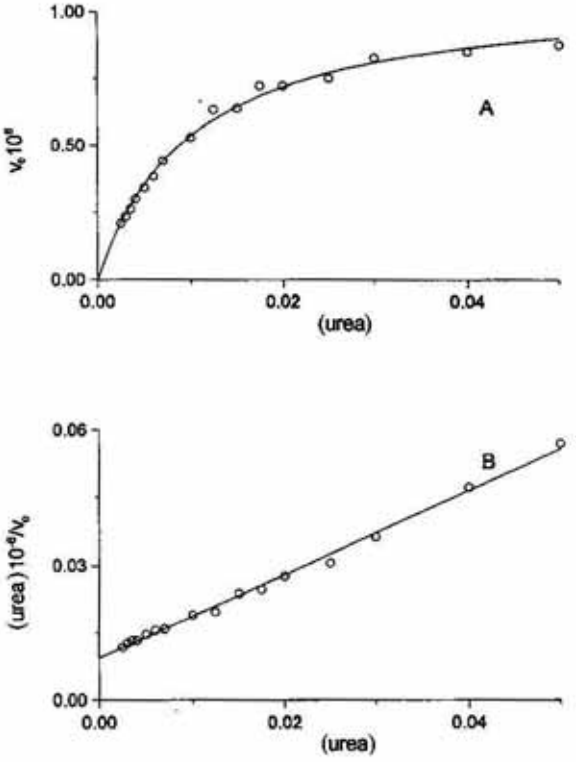

Figure 1. Initial rate of urea hydrolysis by urease as a function of the substrate concentration.

System without inhibitor; v_0 (mol s^{-1}), [urea] (mol dm^{-3}). A, The solid line was calculated by fitting experimental data to Michaelis-Menten equation; B, replot in the Hanes coordinate system.

a higher concentration of phosphate buffer. It is known that phosphates are urease inhibitors of medium strength [16] independently of the presence of other inhibitors.

Experimental values of the initial (v_0) and steady-state reaction rate (v_s)

The results of calorimetric and analytical studies of the hydrolysis of urea at various concentrations of acetohydroxamic acid are shown in Fig. 2 and 3, respectively. For a given time t, the measured and corrected temperature increment $\Delta T'$ (Fig. 2) and analytically determined number of moles of ammonia n_{NH_3} (Fig. 3) are correlated by the following equation:

$$\Delta T' = \frac{1}{2}\frac{\Delta H}{R_{cal}} n_{NH_3} = 74.85\, n_{NH_3} \qquad (11)$$

where $\Delta H = -59580$ J mol^{-1} is the enthalpy of urea hydrolysis [12] and $R_{cal} = 398.00$ J K^{-1} is the heat capacity of the calorimeter.

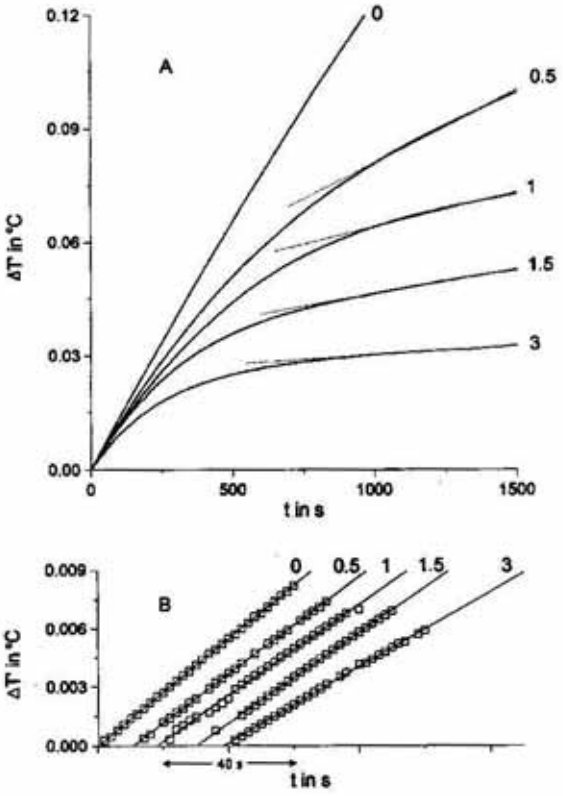

Figure 2. Enthalpimetric progress curves ΔT *vs* t, for urease-catalyzed urea hydrolysis in the presence of acetohydroxamic acid.

Numbers denote [AHA] (mmol dm^{-3}). A, the whole course of the reaction, experimental points are not shown in the plots; B, ΔT *vs* time for the initial stage of reaction.

The initial reaction rate, v_0, at low and moderate inhibitor concentrations was determined from the first ($0 \le t \le 60$ s) linear segment of the calorimetric progress curve (Fig. 2B). Linear least squares analysis yielded the slope (K s^{-1}), which multiplied by the factor: $R_{cal}/|\Delta H| = 398/59580 = 0.006680$ mol K^{-1} yielded the rate v_0 (mol s^{-1}) related to a given inhibitor concentration (Table 1). At high concentrations only the analytical method could be used (Table 2).

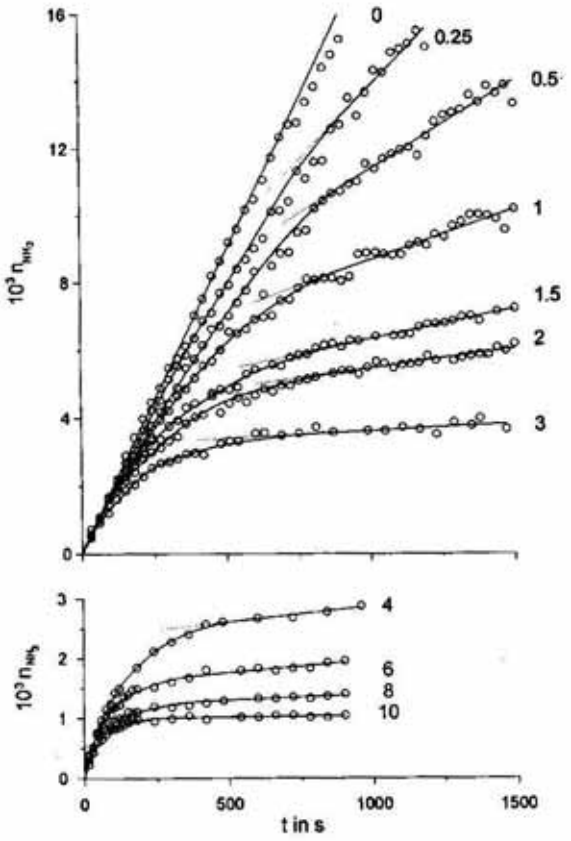

Figure 3. Analytical progress curves for urease-catalyzed urea hydrolysis in the presence of acetohydroxamic acid; n_{NH_3} (mol), moles of ammonia liberated; numbers denote [AHA] (mmol dm^{-3}).

Both techniques were applied to determine the steady-state rate, v_s, in the range of lower concentrations (Table 1). At higher concentrations the slope of the asymptotes and consequently the values of v_s become lower than the experimental error.

Inversion constant (k)

The experimental values of inversion constant k obtained from the progress curves corresponding to all the inhibitor concentrations studied between 0.25 and 10 mmol dm^{-3}, are given in the second column of Table 3.

DISCUSSION

The values of K_m and v_{max} determined in this study for the system without the inhibitor and the experimental data v_0, v_s, k obtained for the systems with the inhibitor allow to calculate the inhibition constants K_i and K_i*. The functional dependence of the constants on inhibitor concentration, K_i, K_i*, k *vs* [I], described in this section, is essential for kinetic analysis of the system.

Inhibition constant of the initial state K_i

The experimental points ([I], v_0) listed in Tables 1 and 2, plotted in the coordinate system $10^{-6}/v_0$ *vs* 10^3 [I] produce a straight line (Fig. 4A) described with eqn. (12):

$$\frac{10^{-6}}{v_0} = 0.1460 \times 10^3\,[\mathrm{I}] + 1.1200 \qquad (12)$$

From eqn. (5) for $[S]_0 = 0.05$ mol dm^{-3} the value K_i was calculated:

$$K_i = 10^{-6} \times 10.2/(0.146 \times 1.075 \times 0.05) = 1.30 \times 10^{-3} \text{ mol dm}^{-3}$$

Table 1. The initial v_0 and steady-state reaction rate v_s at low and medium inhibitor concentrations

10^3 [I] (mol dm^{-3})	$10^6 v_0$ (mol s^{-1})		$10^6 v_s$ (mol s^{-1})		
	Calor.	Anal.	Calor.	Anal.	±error
0.25	–	–	–	0.44	0.06
0.50	0.835	–	0.257	0.285	0.025
1.0	0.782	–	0.124	0.135	0.025
1.5	0.755	–	0.088	0.090	0.006
2.0	–	–	–	0.055	0.006
3.0	0.646	–	0.026	0.023	0.006
4.0	–	0.59	–	0.012	0.006

This value is valid for the whole studied range of inhibitor concentration up to the concentration of 0.010 mol dm^{-3}.

Table 2. The initial reaction rate v_0 and the final ammonia contents n_∞ at high inhibitor concentrations

10^3 [I] (mol dm^{-3})	$10^6 v_0$ (mol s^{-1})	$10^3 n_{NH_3}$ (mol)	
	Anal.	Anal.	± error
4	0.59	0.152	⎫
5	0.54	0.128	⎪
6	0.50	0.112	⎬ 0.006
8	0.44	0.076	⎪
10	0.39	0.067	⎭

Linear dependence of $1/v_0$ on [I] predicted by eqn. (5), observed over the whole range of inhibitor concentration, means that in the initial stage of the reaction (1–2 min) AHA is a classical competitive inhibitor.

Inhibition constant of the steady-state K_i^*

In the range $0<[I]<1$ mmol dm^{-3} the relation, $10^{-6}/v_s$ *vs* 10^3 [I], is linear (Fig. 4B) and given by eqn. (13):

$$\frac{10^{-6}}{v_s} = 5.5814 \times 10^3 [I] + 1.1200 \qquad (13)$$

In this range K_i^* has a constant value. It was determined by comparing the slopes of straight lines (6) and (13) and was found to be:

$$K_i^* = 10^{-6} \times 10.2/(5.5814 \times 1.075 \times 0.05) = 0.0340 \times 10^{-3} \text{ mol dm}^{-3}$$

The values of k_5 and k_6 constants in this inhibitor concentration range were calculated from the transformed eqns. (8) and (9). Using the values:

$$K_i = 1.30 \times 10^{-3}; K_i^* = 0.034 \times 10^{-3}; K_m = 10.2 \times 10^{-3}; [S]_0 = 0.05$$

the following was obtained:

10^3[I]	103 k	$10^3 k_5$	$10^3 k_6$
0.25	0.435 (uncorr)	1.22	0.033
0.5	0.919 (uncorr)	1.66	0.045
1.0	2.04 (corr)	2.15	0.0578

For higher inhibitor concentrations, [I] > 1 mmol dm^{-3}, it was assumed that K_i has a constant value and that $k_6 = 0.0578 \times 10^{-3}$ s^{-1}. From the same eqns. (8) and (9) taking k-corrected, K_i^* and k_5 were calculated.

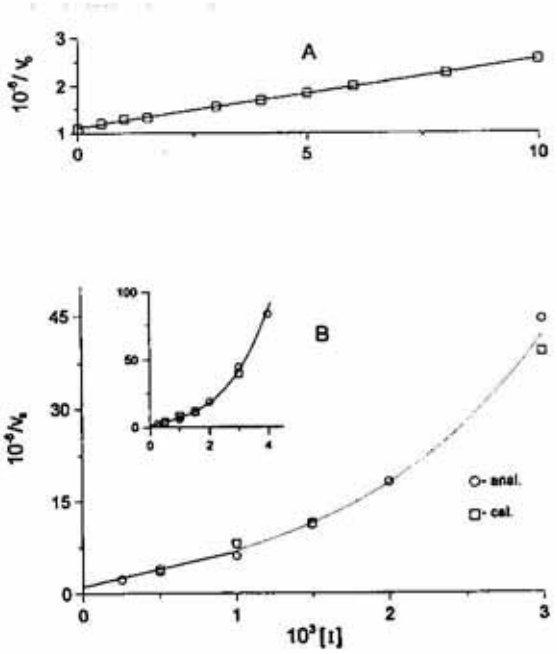

Figure 4. The reciprocal rate of hydrolysis of urea *vs* concentration of acetohydroxamic acid; [I] (mol dm^{-3}).

A, The initial reaction rate v_0 (mol s^{-1}); the ordinate of the first point was calculated from the slope of the line 0 in Fig. 2B; the remaining values were taken from Table 1 and 2. B, The steady-state reaction rate v_s (mol s^{-1}); values ❑, ○, taken from Table 1; points in the range $0 < 10^3$ [I] ≤ 1 determine the line (13). Solid lines are drawn through experimental points. Dotted lines represent eqn. (25).

The values presented in Table 3 show that K_i^* and k_5 do not remain constant. The rate constant k_5 increases with an increase in inhibitor concentration followed by a decrease in K_i^* (eqn. 8) and by an increase in $1/v_s$ faster than predicted by the linear equation (6) (Fig. 4B). Supposedly, the inhibitor

Table 3. The values of the inversion constant k, experimental and calculated from equation $10^3 k = 2.04(10^3[I])^{1.67}$ (see text), of the rate constant k_5, and of the inhibition constant of steady-state K_i^*. The last digit of K_i^* values are given with the accuracy of ±2

10^3 [I] (mol dm^{-3})	$10^3 k$ (s^{-1})		$10^3 k_5$ (s^{-1})	$10^3 K_i^*$ (mol dm^{-3})
	experimental	corrected		
0.25	0.435	–	1.22	0.034
0.50	0.919	–	1.66	0.034
1.0	2.21	2.04	2.15	0.034
1.5	4.40	4.02	3.13	0.024
2.0	6.49	6.49	3.94	0.019
3.0	11.9	12.8	5.34	0.014
4.0	23.7	20.7	6.56	0.011
6.0	41.3	40.7	8.69	0.0086
8.0	70.8	65.7	10.6	0.0071
10.0	88.2	95.4	12.3	0.0061

has an effect on the equilibrium $EI \underset{k_6}{\overset{k_5}{\Leftrightarrow}} EI^*$; the inhibitor shifts the equilibrium to the right, this was neither predicted nor excluded by scheme (1). This question will be further discussed in next sections of this study.

Inversion constant (k)

Having accepted the scheme and assumptions of the theory of slow-binding inhibition and introducing no further assumptions, eqn. (2) describing the reaction progress curve was obtained, which is different from the original version of Morrison & Walsh [10]. Consequently, Morrison's constant k, denoted here as k_{app}, is different from constant k introduced in this study:
$k_{app} = k\,K_i / [I]$. Progress equation (2) is an alternative version of the equation of Morrison & Walsh [10].

The constant k is not connected with any elementary reaction, that is why here it is not called a rate constant. It describes the kinetics of a number of simultaneous reactions proceeding from the initial values [E], [EA], [EI] to the final values $[E]_s$, $[EA]_s$, $[EI]_s$, $[EI^*]_s$. This is why estimation of the inversion constant seems appropriate.

The theory of slow-binding competitive inhibition (10) does not predict any functional dependence of k on [I]. This dependence was found empirically.

The values of $\left(\frac{K_i}{[I]}10^3 k;\ 10^3[I]\right)$ were found lineary related according to eqn. (14):

$$\frac{K_i}{[I]}\,10^3 k = m10^3 + n \qquad (14)$$

where m = 1.16 mol^{-1} dm^3 s^{-1}, and n = 1.90 s^{-1}; correlation coefficient 0.981 (Fig. 5). This means that the points corresponding to the same value of $10^3 k$ and 10^3[I], form a parabola (15):

$$10^3 k = \frac{m}{10^3 K_i}\left(10^3\,[I]\right)^2 + \frac{n}{10^3 K_i}\left(10^3[I]\right) \qquad (15)$$

where $\frac{m}{10^3 K_i} = \frac{1.16}{1.30} = 0.89$ and $\frac{n}{10^3 K_i} = \frac{1.90}{1.30} = 1.46$. The parabola is represented by a solid line in Fig. 6 (the big graph.)

The double logarithmic plot of $10^3 k$ *vs* 10^3 [I] was also linear (insert in Fig. 6):

$$\log(10^3 k) = \bar{m}\log(10^3[I]) + \bar{n} \qquad (16)$$

where $\overline{m}$ = 1.67 and $\overline{n}$ log 2.04 = 0.31; correlation coefficient 0.996.

Equation (16) is an equivalent of function (17):

$$10^3 k = 2.04\,(10^3[\mathrm{I}])^{1.67} \qquad (17)$$

Because of the high value of the correlation coefficient of the line or function (16) eqn. (17) could be used to "smooth" the values k. The obtained k-corrected values were used to calculate K_i* and k_5 constants listed in Table 3. Equation (17) was included into the discussion presented below.

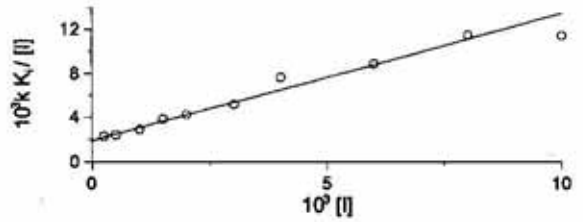

Figure 5. Linearization of the relation: inversion constant k (s^{-1}) *vs* concentration of acetohydroxamic acid; [I] (mol dm^{-3}).

k values taken from Table 3; $K_i = 1.30 \times 10^{-3}$ mol dm^{-3}.

The presented analysis provides evidence that the inhibitor must produce an effect on the inversion constant k, similar to that mentioned in preceding section. The strong dependence of the constant k on its concentration (Table 3) indicates that the inversion EI $\rightarrow$ EI* does not occur spontaneously but with participation of inhibitor molecules according to kinetics of an order of magnitude higher than one.

Supposedly, there occurs reaction (18), in which 'a' is a value to be determined and EI_{a+1} can be identified with EI*:

$$aI + EI = EI_{a+1} \qquad (18)$$

Eqn. (18) can be interpreted in two ways:

1) Eqn. (18) represents the reaction proceeding at a rate given by eqn. (19):

$$-\frac{d[EI]}{dt} = \text{const}\,[I]^a[EI] \qquad (19)$$

or by eqn. (20) simplified to eqn. (21):

$$-\frac{d[EI]}{dt} = k_{app}([EI] - [EI]_s) \qquad (20)$$

$$-\frac{d[EI]}{dt} = k_{app}[EI] \qquad (21)$$

Comparing eqn. (19) with (21) and taking into account that $k_{app} = \frac{kK_i}{[I]}$ the following relationship was found: $k = \frac{\text{const}}{K_i}[I]^{a+1}$, substitution of eqn. (17) with the latter function allowed to calculate: a+1 = 1.67, i.e. a = 2/3.

Since the constant k was determined with a satisfactory accuracy over the whole range of inhibitor concentrations 0.25–10 mmol dm^{-3}, and since the experimental points in this range are well approximated by eq. (17), the value a = 2/3 was considered to be well-founded and will be used in further discussion.

2) Equation (18) represents the equilibrium: $aI + EI \Leftrightarrow EI_{a+1}$, with the equilibrium constant:

$$\frac{[I]^a[EI]_s}{[EI_{a+1}]_s} = \tilde{K} \qquad (22)$$

The following data allow to calculate $\tilde{K}$: for $[I] = 10^{-3}$, $k_5 = 2.15 \times 10^{-3}$ and $k_6 = 0.0578 \times 10^{-3}$; a = 2/3. Hence:

$$\tilde{K} = \frac{k_6}{k_5}[I]^{2/3} = \frac{0.0578}{2.15}10^{-2} =$$
$$= 0.269 \times 10^3\ (\text{mol dm}^{-3})^{2/3}$$

Outside the region of linearity the following relations hold:

$$\frac{k_5}{k_6} = \frac{[I]^{2/3}}{\tilde{K}} \qquad (23)$$

$$\frac{1}{K_i^*} = \frac{1}{K_i}\left(1 + \frac{[I]^{2/3}}{\tilde{K}}\right) \qquad (24)$$

$$\frac{1}{v_s} = \frac{1}{v_{max}}\left[\frac{K_m}{K_i}\left(1 + \frac{[I]^{2/3}}{\tilde{K}}\right)\frac{[I]}{[S]_0} + \left(1 + \frac{K_m}{[S]_0}\right)\right] \qquad (25)$$

A new nonlinear function $1/v_s$ *vs* [I] was obtained. All the parameters in eqn. (25) have constant values over the whole range of inhibitor concentrations from 0.25 to 10 mmol dm^{-3}. After introducing the numerical data eqn. (25) changes into eqn. (26):

$$10^{-6}/v_s = 0.146\,[37.235\,([I] \times 10^3)^{1.67} + + ([I] \times 10^3)] + 1.12 \quad (26)$$

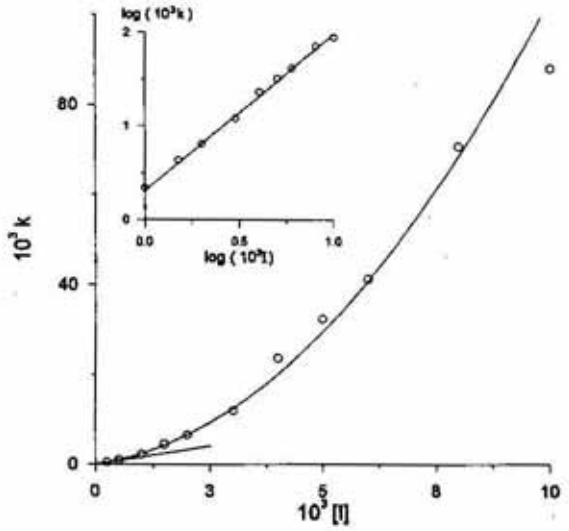

Figure 6. Plot of inversion constant k (s^{-1}) *vs* concentration of acetohydroxamic acid; [I] (mol dm^{-3}).

k Values taken from Table 3. The solid line in the main plot is a representation of eqn. (15): $Y = 0.89\,X^2 + 1.46\,X$. In the insert a double logarithmic plot of the same values (k; 10^3 [I]) is the line (16): $Y = 1.67\,X + 0.31$.

Equation (26) is presented as a dotted line in Fig. 4B. The experimental points, with satisfactory accuracy, are well approximated by eqn. (26).

CONCLUDING REMARKS

An enzymatic system with a competitive slow-binding inhibitor, not disturbed, is characterized by seven constants: [S] k_1, k_2, [I]k_3, k_4, k_5, k_6, k_7 (s^{-1}). In such a system four secondary parameters: K_m, v_{max}, K_i and K_i^* are independent of inhibitor concentration, whereas three: $1/v_0$, $1/v_s$ and k show a linear dependence. Symptoms of disturbance are connected with the constant k_5. It remains invariable at [I] ≤ 1 mmol dm^{-3}. At higher concentrations of the inhibitor, k_5, as the only parameter of the seven given above, does not remain constant. The disturbance is transferred onto the constants K_i^* and k which are directly dependent on k_5. The disturbance of the system can be followed in scheme (1); it occurs in the inversion EI ⇔ EI*. The observed effect of inhibitor concentration on the value of both inversion constant k and rate constant k_5 provides sound evidence that the inactive complex EI* is a product of the reaction: aI + EI = EI_{a+1}. The composition of the complex seems to be independent of the concentration of [I], and the stoichiometric coefficient 'a' is close to 2/3. This leads to the formula (3EI • 2I). Therefore, to inactivate 3 active sites of urease 5 inhibitor molecules are needed. It is probable that the ratio 3:5 corresponds to the scheme (4) presented by Todd & Hausinger in [9]. As the urease active site contains two nickel atoms (confirmed in our laboratory by Olech [17] with the use of EPR), the following coordination systems corresponding to the ratio 3E:5I are possible:

$$\begin{pmatrix} Ni-AHA \\ Ni-AHA \end{pmatrix}\begin{pmatrix} Ni-AHA \\ Ni-AHA \end{pmatrix}\begin{pmatrix} Ni \\ Ni \end{pmatrix}AHA$$

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